The Basic Trypsin Inhibitor of Bovine Pancreas

IX. LOCATION OF THE REACTIVE SITE IN THE CARBOXAMIDOMETHYL DERIVATIVE

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SUMMARY

A derivative of the basic pancreatic inhibitor which contains carboxamidomethyl groups on half-cystine residues 14 and 38 was incubated with trypsin and intact inhibitor at pH 5.5. The carboxamidomethyl derivative after separation from trypsin and intact inhibitor released only lysine when incubated with carboxypeptidase B. The derivative was oxidized with performic acid and a peptide corresponding to residues 1 through 15 in the inhibitor was isolated and identified. It was concluded that complex formation between this inhibitor and trypsin involves cleavage of the peptide bond linking residues 15 and 16 (Lys–Ala).

The mechanism of complex formation between trypsin and soy bean trypsin inhibitor or chicken ovomucoid has been shown to involve cleavage of an arginy1 bond in the inhibitor molecule (2, 3). This has been confirmed for several other trypsin inhibitors (4, 5). Laskowski, Jr. has recently suggested that a single highly exposed lysyl or arginyl residue is a part of the reactive site of most trypsin inhibitors (6).

The only trypsin inhibitor with a known primary structure (7) is the basic pancreatic inhibitor (Kunitz and Northrup's inhibitor). Previous attempts to establish the specific cleavage occurring during complex formation have been unsuccessful, presumably because the modified form reverted to the virgin form too readily. It was predicted (3) that a lysine bond was involved. Two lines of indirect evidence pointed toward the assumption that the bond was Lys 15–Ala 16. Substitutions in the vicinity of Lys 15 by chemical modification of half-cystine residues 14 and 38 modified or abolished inhibitor activity (1, 8). Also, introduction of polyalanine into the ε-amino group of all lysines inactivated the inhibitor (9), but only Lys 15 was not polyalaninated when the inhibitor was combined with trypsin, and the re-isolated inhibitor derivative was active against trypsin.

This communication presents direct evidence that complex formation between trypsin and the carboxamidomethyl derivative (1) of the basic pancreatic trypsin inhibitor results in cleavage of a single lysyl bond in the inhibitor. Chromatographic analysis of the modified inhibitor after performic acid oxidation reveals a peptide corresponding to residues 1 to 15, which indicates that the reactive site of the inhibitor is located at residues 15 and 16 (Lys–Ala).

Reduced, carboxamidomethylated inhibitor was prepared and assayed as described previously (1). The preparation used in these studies was homogeneous as judged by chromatography at pH 6.8 with 0.2 M sodium phosphate on Bio-Rex 70 according to method of Kassell et al. (10). The derivative (51 mg) was made to react at 25° with 190 mg of trypsin previously treated with tosyl phenylalanyl chloromethyl ketone (11). The total volume was 7.0 ml of 0.05 M Tris, 0.2 M NaCl, pH 8.0, at a rate of 22 ml per hour; 6.0 ml fractions were collected.

FIG. 1. Gel filtration of the trypsin-inhibitor reaction mixture on Sephadex G-50. The mixture was placed on a column (2.5 X 90 cm) and eluted at 25° with 0.01 M Tris-0.2 M NaCl, pH 8.0, at a rate of 22 ml per hour; 6.0-ml fractions were collected.

The addition of intact inhibitor, which forms a more stable complex with trypsin than does the carboxamidomethyl derivative, was based on the assumption that the presence of an excess of intact inhibitor would suppress trypsin autolysis and, more importantly, prevent any random digestion of the carboxamidomethyl inhibitor. If the postulated Lys 15–Ala 16 cleavage occurred in the carboxamidomethyl derivative during complex formation, then the products of the above reaction mixture should be: trypsin-intact inhibitor complex, a slight excess of intact inhibitor, virgin carboxamidomethyl inhibitor, and modified carboxamidomethyl inhibitor (peptide bond cleaved). The amount of modified carboxamidomethyl inhibitor would, of course, be dependent upon the rate of conversion of virgin to modified form and the rate of dissociation of modified form from trypsin under the experimental conditions.

After 7 days the solution was titrated to pH 8.0 with NaOH and centrifuged. The clear supernatant was chromatographed on Sephadex G-50 as shown in Fig. 1. Peak I corresponded to

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§The terminology of Ozawa and Laskowski, Jr. (3) is used throughout.

§§ Obtained through the courtesy of Dr. G. Haberland of Farbenfabriken Bayer AG, Leverkusen-Bayerwerk, Germany.

Unpublished observations.
trypsin-intact inhibitor complex; Peaks II and IV consisted of trypsin autolysis peptides. The position of Peak III corresponded to that of the carboxamidomethyl derivative in a control experiment (not shown). The material in Peak III was dialyzed against water, lyophilized, dissolved in 0.2 M sodium phosphate, pH 6.83, and chromatographed on Bio-Rex 70 as shown in Fig. 2. Peak I emerged in the same position as did tryptic autolysis products in a control experiment run under identical conditions (not shown). Peak II was eluted in the position expected for carboxamidomethyl inhibitor. It was concentrated, dialyzed against water, and lyophilized. Amino acid analysis indicated that this peak corresponded to carboxamidomethyl inhibitor contaminated with about 9% of intact inhibitor as judged by the slightly low yield of carboxymethyl cysteine. Thus, over a period of 1 week in the presence of intact inhibitor, essentially all of the carboxamidomethyl derivative was displaced from trypsin.

An aliquot of the material of Peak II (4.3 mg) corresponding to 0.66 μmole of inhibitor was dissolved in 6.0 ml of 0.04 M sodium borate, pH 7.5, and allowed to react with 0.43 mg of carboxypeptidase B (Worthington, diisopropyl fluorophosphate-treated) for 24 hours at 23°. No free arginine could be detected with the amino acid analyzer. However, 0.11 μmole of lysine was found, or about 17% of the total expected if all of the carboxamidomethyl inhibitor molecules had had a single lysine bond cleaved. Assay of an aliquot of Peak II from Bio-Rex 70 (Fig. 2) showed that the inhibitor had lost 17 to 20% of its original activity as a result of incubation with trypsin. No further decrease in activity was noted after carboxypeptidase B treatment. Thus, it appeared that most (about 80%) of the inhibitor derivative had dissociated from trypsin without modification. However, the amount of lysine released in the carboxypeptidase B reaction was almost identical with the amount of activity lost during incubation with trypsin, and corresponded to approximately 17% in both cases.

A plausible explanation for the inactivity of the modified inhibitor, when all other known modified inhibitors are active, is that removal of the neighboring disulfide bridge 14-38 made it impossible for the modified inhibitor to assume the proper conformation for activity.

The remainder of the material of Peak II was oxidized with performic acid (12), lyophilized, dissolved in 0.01 N HCl, and chromatographed on Sephadex G-25 as shown in Fig. 3. Peak I was assumed to be a mixture of oxidized inhibitor, the large core peptide from the modified inhibitor, and possibly some tryptic autolysis peptides. It was not analyzed further. The composition of Peaks III and IV did not correspond to any segments of the known sequence of the pancreatic inhibitor. Since these peaks also had relatively high amounts of histidine, serine, and

![Fig. 2. Chromatography of Peak III from Fig. 1 on Bio-Rex 70. The material from Peak III in Fig. 1 was dissolved in 3.0 ml of 0.2 M sodium phosphate, pH 6.83. The column (1.5 X 25 cm) was eluted with the same buffer at 20 ml per hour and 4.0-ml fractions were collected.](http://www.jbc.org/)

![Fig. 3. Gel filtration of Peak II from Fig. 2 on Sephadex G-25. The performic acid oxidized material from Peak II in Fig. 2 was dissolved in a few drops of concentrated HCl and the volume was adjusted to 5.0 ml with water. The column (2.5 X 40 cm) was eluted with 0.01 N HCl at 38 ml per hour and 4.0 ml fractions were collected.](http://www.jbc.org/)

**TABLE I**

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Residues per molecule</th>
<th>Amino acid</th>
<th>Residues per molecule</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>1.05</td>
<td>Glycine</td>
<td>1.00</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.71</td>
<td>Alanine</td>
<td>0.25</td>
</tr>
<tr>
<td>Cysteic acid</td>
<td>1.06*</td>
<td>Half-cystine</td>
<td>0.23</td>
</tr>
<tr>
<td>Carboxymethyl cysteine</td>
<td>0.09</td>
<td>Valine</td>
<td>0</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>1.01</td>
<td>Methionine</td>
<td>0</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.90</td>
<td>Isoleucine</td>
<td>0.03</td>
</tr>
<tr>
<td>Serine</td>
<td>0.05</td>
<td>Leucine</td>
<td>0.82</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>0.94</td>
<td>Tyrosine</td>
<td>0.84</td>
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<tr>
<td>Proline</td>
<td>3.60</td>
<td>Phenylalanine</td>
<td>0.93</td>
</tr>
</tbody>
</table>

* The oxidation product of residue 14 was apparently not identifiable in the above system. A similar low recovery has previously been noted (see Table 3 in Reference 7).
glutamic acid, it was concluded that they represented tryptic autolysis products which had co-eluted with the inhibitor on Bio-Rex (Fig. 2).

Amino acid composition of Peak II from Sephadex G-25 corresponded to residues 1 through 15 (cf. Reference 7) in the sequence of the inhibitor (Table I). It was concluded that incubation of the carboxamidomethyl derivative of the basic pancreatic inhibitor with trypsin had resulted in the cleavage of a single peptide bond at lysine 15. The reactive site of this inhibitor, therefore, involves residues 15 and 16 (Lys–Ala).

REFERENCES

Occurrence of Glycylhydroxyprolyl Sequences in Earthworm Cuticle Collagen*

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SUMMARY

Salt-soluble collagen of earthworm cuticle contains much hydroxyproline in the second position of tripeptide sequences beginning with glycine, as determined by sequence analysis of mixed peptides released by the action of clostridial collagenase. A tripeptide, Gly–Hyp–Ala, was isolated from these digests and identified by sequence analysis and by comparison with a synthetic reference peptide. It was estimated to account for approximately 10% of the hydroxyproline of both purified salt-soluble collagen and of the total collagen of the cuticle.

Hydroxyproline, according to current understanding (1), is synthesized by the hydroxylation of certain proline residues in a polypeptide precursor of collagen. Enzymes catalyzing proline hydroxylation have been purified from chick embryo (2, 3) and detected in a number of other tissues (4); substantial information has also accrued concerning some of the properties of natural or synthetic proline-containing polypeptides that permit substrate activity for enzymatic hydroxylation (4–7).

A major question concerns the specificity of proline hydroxylation: are specific prolines in the precursor peptide reproducibly hydroxylated in a population of molecules, and if so, what factors determine the selection of residues for hydroxylation? The evidence bearing on this question suggests a simple generalization: that proline residues in position 3 of the repeating triplet amino acid sequences in collagen in which glycine occupies position 1 can be hydroxylated; prolines in position 2 cannot be. Thus, the sequence of specific peptides derived from bovine Achilles tendon (8), data on Edman-sequencing of mixed peptides resulting from collagenase-digested call skin collagen (9) or ichthyocol (10), and more recent observations on the enzymatic hydroxylation of proline in specific positions either in synthetic (11) or natural (12) substrates, are all consistent with this hypothesis. All proline residues in position 3 may not, however, be hydroxylated even in mature collagen, as suggested by the recent work of Bornstein (13).

The data suggesting that prolines susceptible to hydroxylation must be in position 3 relative to glycine are based on vertebrate collagens. Because a specificity restriction of such simplicity might well operate very generally in the hydroxylation of collagens from many forms, and because earthworm cuticle collagen contains a uniquely high hydroxyproline to proline ratio (14), we have examined this collagen for the possible presence of hydroxyproline in positions immediately following glycine, the position which appears interdicted for hydroxylation in vertebrate collagens. Our findings indicate that significant amounts of hydroxyproline appear to occupy position 2, both on the basis of a sequence analysis of mixed collagenase-released peptides, and by the isolation of Gly–Hyp–Ala as a significant component of the cuticle collagen.

Earthworms were obtained either from Wholesale Bait Company (Hamilton, Ohio) or from Carolina Biological Supply (Burlington, South Carolina). Salt-soluble cuticle collagen was isolated by the method of Josse and Harrington (15); the final ammonium sulfate precipitate was taken up in one-fourth the original volume of 0.2 M NaCl and dialyzed exhaustively against water. Amino acid analysis (hydrolysis in constant boiling HCl under nitrogen in sealed tubes at 110° for 17 hours, analysis with the standard Technicon system) gave results in general agreement with other published data (14–18). Glycine represented approximately 33% of the residues; hydroxyproline, 16%; alanine, 10%; serine, 9%; and glutamic acid, 8%; the hydroxyproline to proline ratio averaged about 16.

To examine the position of hydroxyproline relative to glycine, the purified cuticle collagen was treated with collagenase since, with vertebrate collagens, this enzyme releases a preponderance of short peptides with NH₂-terminal glycine (10). Preliminary trials indicated that heat-denatured cuticle collagen was a much better substrate for clostridial collagenase than was the native collagen. Conditions were found in which collagenase rapidly released all the hydroxyproline as peptides soluble in 75% ethanol. To obtain a mixture of peptides suitable for sequence analysis, collagen preparations were incubated at 37° for 90 min with purified commercial collagenase (CLSVA, Worthington). Incubation mixtures contained 1 to 2 mg of collagen per ml, one-fifth this weight of collagenase, 5 mM CaCl₂, and 50 mM Tris, pH 7.4. The reaction was stopped by adding 3 volumes of

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